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Nerve Growth Factor Gene Expression in the Developing Rat Brain

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The regulation of nerve growth factor (NGF) protein and NGF messenger RNA (mRNA) in the developing rat brain has been studied to assess the hypothesis that NGF supports the differentiation of cholinergic neurons in the basal forebrain. In the adult, the major targets of these neurons, the hippocampus and neocortex, contain the highest concentrations of NGF mRNA, but comparatively low ratios of NGF protein to its mRNA. In contrast, a high concentration of NGF protein and a low concentration of NGF mRNA were seen in the basal forebrain, consistent with retrograde transport of NGF protein into this region from the neocortex and hippocampus. In these two target regions NGF and NGF mRNA were barely detectable at birth, their concentrations increased to a peak at day 21, and then NGF mRNA, but not NGF protein, declined threefold by day 35. NGF accumulation in the basal forebrain paralleled that in the target regions and preceded an increase in choline acetyltransferase, suggesting that the differentiation of cholinergic projection neurons is indeed regulated by retrogradely transported NGF. In addition, high ratios of NGF protein to NGF mRNA, comparable to that in the basal forebrain, were seen in the olfactory bulb and cerebellum, suggesting that NGF may be transported into these regions by unidentified neurons.

THE RELEASE OF DIFFUSIBLE TROPHIC factors by nerve cells and their targets has been thought to play a major role in the development and maintenance of connections in the nervous system (*1*). Although a number of putative trophic factors have been identified, only nerve growth factor (NGF) has been shown to be essential for neuronal survival in vivo, where it is required for the development of sympathetic and sensory neurons in the peripheral nervous system (*2*). Neurons in the central nervous system (CNS) may also depend on NGF during development since recent work has shown that the rat brain contains NGF (*3*), NGF messenger RNA (mRNA) (*3, 4*), NGF receptors (*5*), and NGF-responsive neurons (*6, 7*). Several lines of evidence suggest that one function of NGF in the CNS is to regulate the differentiation of the cholinergic projection neurons found within various nuclei of the basal forebrain. The hippocampus and neocortex, two regions innervated by these neurons (*8*), contain the highest levels of NGF protein and NGF mRNA found in the brain (*3, 4*). [^{125}I]-labeled NGF injected into cortical and hippocampal regions is transported retrogradely to the nucleus basalis (*9*) and the medial septum–diagonal band region (*10*), respectively. In neonatal rats, intraventricular injections of NGF increase choline acetyltransferase (ChAT) activity, the enzyme responsible for acetylcholine synthesis, in the basal forebrain (*11*). If NGF regulates cholinergic differentiation, then the target regions should be capable of supplying NGF to cholinergic fibers during the period of innervation. Our results show that the developmental increase in NGF content of the basal forebrain parallels that of the neocortex and hippocampus and precedes an increase of similar magnitude in ChAT activity, consistent with regulation of cholinergic differentiation by target-derived NGF.

NGF mRNA was measured in five brain regions of adult Sprague-Dawley rats by blotting polyadenylated [poly(A)$^+$] RNA onto nitrocellulose paper and hybridizing with a single-stranded NGF complementary DNA (cDNA) probe (*12*). NGF protein was measured by a two-site enzyme-linked immunosorbant assay (ELISA) with goat polyclonal and monoclonal antibodies to β-NGF (*13*). The hippocampus and neocortex contained relatively high amounts of both NGF and NGF mRNA (Table 1), in agreement with previous reports (*3, 4*). Although there also were large amounts of NGF in the basal forebrain, there was five times less NGF mRNA than in target regions. This is consistent with retrograde transport of NGF from the neocortex and hippocampus to the basal forebrain by cholinergic projection neurons (*9, 10*).

The hypothesis that NGF in the neocortex and hippocampus could provide trophic support for developing basal forebrain neurons was tested by comparing the increase in NGF content and ChAT activity of these regions during postnatal development. Because the neocortex and hippocampus con-

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tain few intrinsic cholinergic neurons (*14*), the differentiation of presynaptic cholinergic terminals in these regions can be followed by measuring increases in ChAT activity. The total amount of NGF in the neocortex (Fig. 1A) and hippocampus (Fig. 1B) increased rapidly after day 10, reached a peak by day 21, and then declined 20 to 30% before rising to adult values. The time course of increase in NGF content of the basal forebrain closely paralleled that of the two target regions (Fig. 1C). Accumulation of NGF in the basal forebrain appeared to result primarily from retrograde transport from target regions, rather than from local synthesis. The amount of NGF mRNA in the basal forebrain between day 7, the earliest age tested, and adult remained constant, while NGF protein content increased by three to four times during this period. The increase in NGF content of all three regions also preceded an increase of similar magnitude in ChAT activity by several days (Fig. 1).

The regulation of NGF in cholinergic target regions was investigated by comparing NGF and NGF mRNA content during postnatal development. The increase in NGF mRNA in the rat neocortex indicated that gene expression is developmentally regulated by control of transcription or mRNA stability (Fig. 2A). NGF mRNA content of the neocortex and hippocampus, expressed as percent of adult, increased from approximately 10% on day 1 to a peak of 300 to 400% by day 21, and subsequently decreased by day 35 to adult values (Fig. 2B). Early in postnatal development, the amount of NGF protein in target regions appeared to be determined by the amount of NGF mRNA, as the increase in NGF roughly paralleled that of its mRNA during the first 4 postnatal weeks (Fig. 2C). Thereafter, the amount of NGF protein increased slightly while NGF mRNA decreased, suggesting that there is a change in the regulation of NGF expression in these two targets late in development.

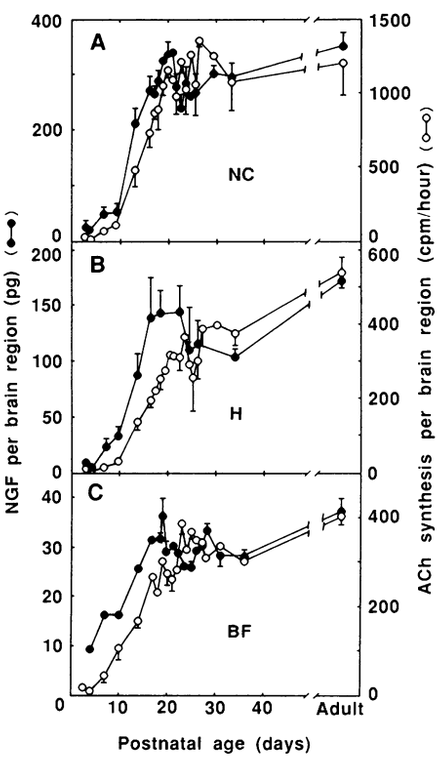

Fig. 1. Postnatal increase in NGF content and choline acetyltransferase activity of (A) the neocortex (NC), (B) the hippocampus (H) and (C), and the basal forebrain (BF). Data are expressed as total activity per brain region to control for variability in dissection of the basal forebrain. NGF protein was measured by two-site immunosorbant assay (*13*). ChAT activity was determined by the synthesis of [^{3}H]acetylcholine (*25*). Each point represents the mean ± SEM of three to six determinations.

The development of NGF expression is not synchronous throughout the rat brain. The amount of NGF and NGF mRNA in the cerebellum, which receives little or no cholinergic innervation (*8, 14*), did not reach a peak during the third postnatal week (Fig. 3). In contrast to the neocortex and hippocampus, the amounts of the protein and the mRNA decreased in parallel from day 10 to adult. Differences between the developmental regulation of NGF expression in cholinergic target regions and the cerebellum are also indicated by a comparison of the molar ratio of NGF protein to its mRNA at different ages (Fig. 4). The ratio in the neocortex and hippocampus remained relatively low early in postnatal development. Later in development there was a change in this ratio as a given amount of NGF mRNA resulted in two to three times as much NGF protein in the adult compared to the neonate. In contrast, the molar ratio of NGF protein to NGF mRNA in the cerebellum remained consistently high between day 10 and adult.

The high concentration of NGF relative to its mRNA in the adult cerebellum and olfactory bulb (Table 1), which contain few if any cholinergic neurons capable of retrogradely transporting NGF from other brain regions (*8, 14, 15*), suggests that NGF also may act as a trophic factor for noncholinergic neurons in the CNS (*4, 7*). The amount of NGF in the adult cerebellum appears to be greater than that necessary to support the sympathetic innervation of the vasculature (*4*). In the olfactory bulb, the molar ratio of NGF to its mRNA was expected to be low because this region, like the neocortex and hippocampus, is innervated by cholinergic fibers from the basal forebrain (*8, 14, 15*). However, the ratio was five to eight times higher than in the neocortex and hippocampus. The relatively high ratios of NGF protein to its mRNA in olfactory bulb and cerebellum may be due to net transport of NGF into these regions. For example, mitral and tufted cells from the olfactory bulb project to the anterior olfactory nucleus, piriform cortex, and entorhinal cortex (*15*), regions rich in NGF and NGF mRNA (*4, 16*). Alternatively, NGF synthesized within the olfactory bulb and cerebellum may accumulate locally rather than be transported out. Accumulation of NGF in the adult olfactory bulb may be important for the

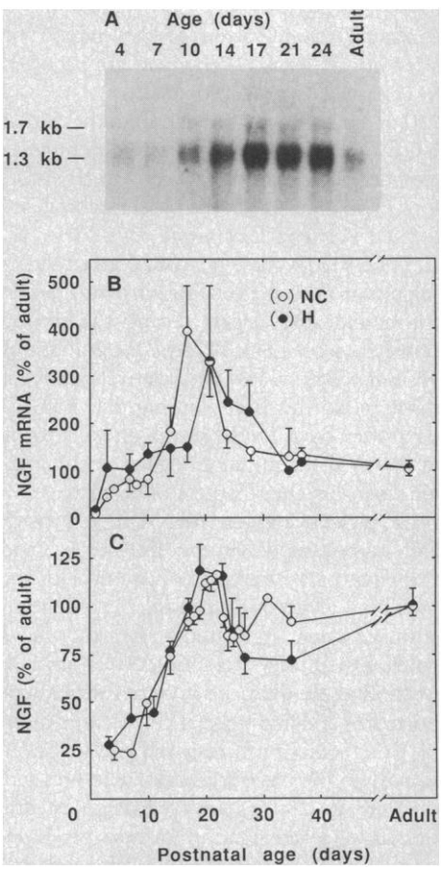

Fig. 2. NGF and NGF mRNA in neocortex and hippocampus during postnatal development. (A) Blot analysis of poly(A)$^+$ RNA from neocortex hybridized with [^{32}P]-labeled NGF cDNA probe. Amounts of poly(A)$^+$ RNA loaded per lane: Day 4, 5.0 μg; day 7, 5.0 μg; day 10, 3.8 μg; day 14, 5.0 μg; day 17, 5.0 μg; day 21, 4.0 μg; day 24, 3.8 μg; adult, 5.0 μg. (B) NGF mRNA in developing neocortex (NC) and hippocampus (H). For each age, NGF mRNA content is normalized to poly(A)$^+$ RNA and expressed as percent of adult. Data points represent the mean ± SEM of three to nine (neocortex) or two to five (hippocampus) independent determinations. (C) NGF protein in developing neocortex and hippocampus. Data from Fig. 1, A and B, are normalized to tissue wet weight and expressed as percent of adult. Each point represents the mean ± SEM of three to six determinations. Although the development of NGF mRNA appears to lag behind that of NGF protein in the hippocampus, this apparent lag is not statistically significant.

Table 1. NGF and NGF mRNA in adult rat brain. NGF protein and NGF mRNA are expressed as mean ± SEM, and the number of independent determinations is in parentheses. The cholinergic nuclei of the basal forebrain were obtained by removal of the overlying cortex and the striatum and dissection of the forebrain situated posterior to the olfactory nucleus and anterior to the hypothalamus. The molar ratios of NGF protein to mRNA were calculated by utilizing an average value of 0.01 μg of poly(A)$^+$ RNA per milligram of tissue and molecular sizes of 13 kD and 429 kD for β-NGF and 1.3-kb NGF mRNA, respectively.

Region	NGF protein (fg/mg tissue)	NGF mRNA [fg/μg poly(A)$^+$ RNA]	Ratio (NGF/NGF mRNA)
Hippocampus	1300 ± 160 (10)	650 ± 70 (4)	6,600
Neocortex	750 ± 70 (10)	590 ± 80 (9)	4,200
Basal forebrain	660 ± 60 (7)	90 ± 30 (4)	24,000
Olfactory bulb	880 ± 10 (3)	110 ± 20 (4)	26,000
Cerebellum	160 ± 20 (3)	20 ± 10 (4)	26,000

continual reinnervation of this region by olfactory neurons (*17*), which are reported to contain NGF receptors (*18*).

Our findings support the hypothesis that NGF is a trophic factor for differentiating cholinergic projection neurons in the basal forebrain. Neurons in the basal forebrain are formed between embryonic days 13 to 17, and fibers from these neurons contact target regions around the time of birth and ramify throughout the targets during the second postnatal week (*19*). Because the amount of NGF is relatively low and relatively uniform throughout the brain during the first 10 days after birth, NGF seems unlikely to be involved in the initial guidance of cholinergic fibers to their targets. The increase in NGF protein and its mRNA in neocortex and hippocampus during the second and third postnatal weeks is consistent with the possibility that NGF supports the differentiation of cholinergic fibers after they have entered their target regions. A role for NGF in cholinergic differentiation is further supported by the finding that the accumulation of NGF protein precedes the rise in ChAT activity in the basal forebrain. The presence of NGF in this region appears to be due primarily to retrograde transport by developing fibers, rather than local synthesis, because the postnatal increase in NGF protein is not accompanied by an increase in NGF mRNA. In the adult basal forebrain, the high ratio of NGF protein to NGF mRNA indicates that mature neurons continue to transport significant amounts of NGF. Intraventricular injections of NGF rescue cholinergic neurons in the basal forebrain that otherwise would degenerate following transection of their axons (*20*). Thus, NGF also may be important for the maintenance of mature cholinergic neurons.

The developmental increase in the ratio of NGF protein to its mRNA, which appears to occur specifically in cholinergic target regions, could reflect changes in NGF expression or changes in the clearance of NGF from target tissues by retrograde transport, diffusion, or degradation. In addition, alternate pathways for mRNA splicing (*21*) and precursor processing (*22*) could result in NGF-containing peptides that are not detected by the ELISA (*23*). The mechanisms that regulate NGF expression in cholinergic target tissues are at present unclear. The observed decrease in NGF mRNA levels after day 21 in the neocortex and hippocampus, but not cerebellum, suggests that cholinergic fibers may play a role in regulating NGF mRNA levels during development. However, in adults, innervating fibers from NGF-dependent neurons do not appear to modulate NGF mRNA levels (*24*). Therefore, if cholinergic neurons regulate NGF expression in targets during development, they must act by mechanisms that are no longer effective in the adult.

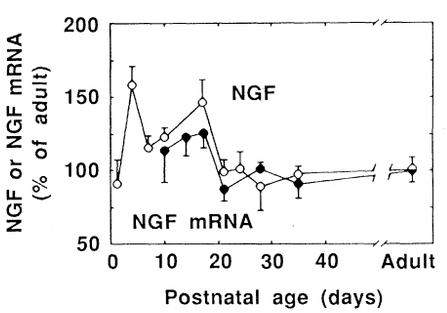

Fig. 3. NGF expression in cerebellum during postnatal development. NGF and NGF mRNA levels are expressed as in Fig. 2. NGF and NGF mRNA data represent the mean ± SEM of six to nine and three to five determinations, respectively.

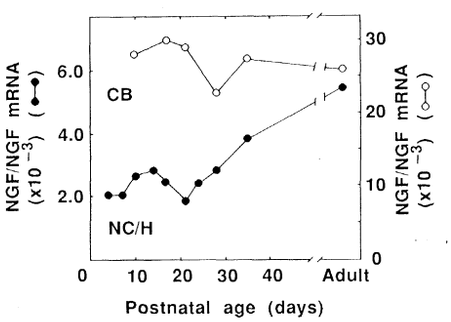

Fig. 4. Molar ratios of NGF protein to NGF mRNA during development. The hippocampus and neocortex data from Fig. 2 were combined and the average value for NGF protein was divided by that for NGF mRNA. The ratios for cerebellum were calculated from the data in Fig. 3.

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12. Samples from several animals were pooled to yield 0.5 to 1 g of wet weight tissue. Purification of poly(A)$^+$ RNA and RNA blot assays were performed as described [D. L. Shelton and L. F. Reichardt, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 7951 (1984)]. NGF mRNA was detected with a [^{32}P]-labeled single-stranded cDNA probe (*21*). The minor 1.7-kb band and the major 1.3-kb band were combined during densitometric scanning to provide an estimate of total NGF mRNA. The relative proportions of these two bands remained unchanged during development. All gels contained as standards NGF RNA synthesized in vitro with SP6 RNA polymerase (Promega Biotech) and a plasmid containing the SP6 promoter upstream from the PST I fragment of the NGF cDNA. The use of NGF RNA as standards, rather than M13 template or submaxillary gland poly(A)$^+$ RNA, may account for the approximately fourfold higher concentration of NGF mRNA in the brain observed here as compared to (*4*). The amount of poly(A)$^+$ RNA loaded per lane was confirmed by washing off the NGF probe and rehybridizing with [^{32}P]-labeled oligo(dT). During postnatal development, poly(A)$^+$ RNA as a percent of total brain RNA increased from approximately 1.5 to 2.3%.
13. The NGF ELISA was performed as described (G. Weskamp and U. Otten, *J. Neurochem.*, in press). Tissue samples from individual rats were sonicated (100 to 150 mg of wet weight tissue per milliliter) in 10 m*M* phosphate buffer, *p*H 7.4, containing 400 m*M* NaCl, 0.5% bovine serum albumin, 0.05% Tween 20 and the protease inhibitors phenylmethylsulfonyl fluoride (0.1 m*M*), aprotinin (20 kallikrein units per ml) and benzethonium chloride (0.1 m*M*). Aliquots were removed for determination of ChAT activity and the remainder was centrifuged at 17,000*g* for 1 hour. The supernatants were applied

to 96-well plates (Nunc 1) that had been coated with goat polyclonal antibodies to NGF (specific) or normal goat serum (nonspecific). The immobilized NGF was then incubated with monoclonal antibody 23C4 to β-NGF, and the complex was visualized by sequential reactions with biotin-conjugated goat antibody to rat IgG, horseradish peroxidase–conjugated strept-avidin, and the peroxidase substrate ortho-phenylenediamine. The absorbance of the wells was read at 492 nm. The amount of NGF in individual samples was calculated by comparison with β-NGF purified from mouse submaxillary gland. The sensitivity of the assay, defined as two times the background signal, was routinely 0.1 to 1 pg of NGF per assay. Recovery of NGF from the neocortex and hippocampus, determined by addition of purified mouse β-NGF to the homogenates followed by ELISA, was greater than 75%. The data were not corrected for recovery.

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23. The antibodies used in the ELISA do not recognize either the 34-kD or 28-kD precursors synthesized in vitro.
24. Selective removal of sympathetic and sensory fibers does not alter NGF mRNA levels in the adult rat iris [D. L. Shelton and L. F. Reichardt, *J. Cell Biol.* **102**, 1940 (1986)]. Similarly, in the adult CNS, destruction of cholinergic fibers innervating the hippocampus by fimbria-fornix lesions does not change NGF mRNA levels in this target (*3*) [S. Korsching, R. Heumann, H. Thoenen, F. Hefti, *Neurosci. Lett.* **66**, 175 (1986)], although NGF synthesis appears to be stimulated within the medial septal nucleus, which contains the denervated neurons (G. Weskamp, U. E. Gasser, A. R. Dravid, U. Otten, *Neurosci. Lett.*, in press).
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An Early Event in the Interferon-Induced Transmembrane Signaling Process

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Human interferon stimulates a transient two- to threefold increase in the concentration of diacylglycerol and inositol tris-phosphate within 15 to 30 seconds of cell exposure to interferon. Antibodies to interferon inhibit this effect. The stimulation was measurable in isolated cell membranes exposed to interferon. Human α and β, but not γ, interferon stimulate this increase in cells containing the appropriate interferon receptor. The effect was proportional to the number of interferon receptors. Both the diacylglycerol increase and antiviral effects induced by interferon could be correlated in terms of dose dependence. Thus, a transient diacylglycerol increase is an early event in the interferon-induced transmembrane signaling process.

INTERFERON (IFN) IS ANTIVIRAL, HAS immunoregulatory effects in living cells, and inhibits cell growth (*1*). Clinically, it is an effective prophylactic agent against the common cold (*2*) and has an antiviral effect in other viral diseases of man such as warts, hepatitis B, and reactivated herpes simplex (*3*). It is reported to have an antitumor effect in hairy-cell leukemia, juvenile laryngeal papilloma, and intraepithelial neoplasia of the uterine cervix (*4*). However, little is known of the physiological role of IFN and of the mechanism by which IFN transmits its effects from the cell surface into the cell where its actions are manifested. We undertook to study the early events that occur in cells seconds after they are exposed to IFN to identify the changes associated with the transmembrane processing of the IFN signal. For this reason, we chose to study primary human diploid fibroblasts and Daudi cells because they are most commonly used in IFN assays and in studying the binding of human IFN's α and β to its putative receptor (*5*).

Primary human fibroblasts, Daudi cells, and isolated cell membranes were incubated at 37°C with human IFN's α, β, or γ for various times. The cells were immediately extracted and assayed for diacylglycerol and inositol phosphates. The treated cells were also assayed for changes in cytosolic-free Ca^{2+} by means of quin-2 and fura-2 as fluorescent indicators (*6*). There were immediate increases in diacylglycerol and inositol bis- and tris-phosphates in human fibroblasts within 30 seconds after exposure to human IFN's α, β, and γ (Fig. 1A). The concentration of diacylglycerol returned to basal level within 30 to 60 seconds. The concentrations of inositol bis- and tris-phosphates returned to basal levels by 10 minutes. Similar results were observed in Daudi cells except that the rise in diacylglycerol concentration was faster in cells treated with human IFN α than with human IFN β (Fig. 1B). The kinetics of this response to human IFN α was about 15 seconds faster than those of IFN β. A statistically significant increase (49%) in diacylglycerol was observed in isolated Daudi cell membranes exposed to human IFN β (Table 1), suggesting that the increase in diacylglycerol concentration occurs in the cell membrane

Table 1. Diacylglycerol levels in different cell types and in cell membranes exposed to human IFN's α, β, and γ; N.D., not determined.

Cells* or membranes†	Diacylglycerol (% of control) after treatment with IFN		
	α	β	γ
Human diploid fibroblasts	280 ± 21‡	281 ± 25‡	343 ± 14‡
Daudi cells	202 ± 3‡	352 ± 11‡	98 ± 2
Mouse A_9 cells	104 ± 7	111 ± 21	96 ± 7
Mouse A_9 × human WaV hybrid	146 ± 2‡	156 ± 13‡	102 ± 2
Mouse A_9 × human WaIII hybrid	N.D.	101 ± 31	N.D.
WaV subclone (WaVR4dF94a)	187 ± 13‡	201 ± 6‡	97 ± 1
Daudi cell membranes	N.D.	149 ± 12‡	N.D.

*Fibroblast cells were grown to confluency (2×10^4 to 4×10^4 per square centimeter) in 75-cm^2 flasks. Daudi cells in 5-ml suspensions were at a density of 1×10^6 cells per milliliter. The cells were exposed to human IFN's α, β, and γ (4000 IU/ml) for 30 seconds and then extracted and assayed for diacylglycerol by the method of Habenicht *et al.* (*14*). †Membranes from Daudi cells (5×10^6) prelabeled with [2-^{3}H]glycerol and prepared by the method of Lucas *et al.* (*19*) were incubated for 10 minutes at 37°C with human IFN β (10,000 IU/ml). ‡Indicates that the value shown is statistically different from the untreated control by Student *t* test at $P < 0.05$. Each value is the mean ± SD of duplicates.

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